Cutting Edge: Hematopoietic-Derived APCs Select Regulatory T Cells in Thymus

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Cutting Edge: Hematopoietic-Derived APCs Select Regulatory T Cells in Thymus

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Recognition of self-peptide–MHC complexes by high-affinity TCRs and CD28 signaling are critical for the development of forkhead-winged helix box transcription factor 3+ regulatory T cells (Tregs) in thymus. However, the type of APCs that are responsible for selecting Tregs has remained unclear. To dissect the role of hematopoietic-derived APCs (HCs) and thymic epithelial cells (TECs) in Treg selection, we constructed bone marrow chimeras with disrupted CD28/B7 signaling in the HC or TEC compartment and analyzed the generation of Tregs in the thymus. We found that both HCs and TECs were independently able to fully reconstitute the Treg population in the thymus of bone marrow chimeras. In addition, Treg selection requires the TCR signal and CD28 costimulation presented in cis on the same APC type in vivo. This study demonstrates a new role, to our knowledge, for HCs in the development of Tregs in thymus. The Journal of Immunology, 2010, 185: 3819–3823.

Forkhead-winged helix box transcription factor 3 (Foxp3)+ regulatory T cells (Tregs) develop in the thymus and are required for the maintenance of self-tolerance (1). Tregs develop from thymocytes with high affinity for self-peptide–MHC complexes (2) and require CD28/B7 signaling for their generation, peripheral homeostasis, and functional activation (3–5). Two types of thymic APCs, thymic epithelial cells (TECs), and dendritic cells (DCs), express B7 molecules and have the capacity to present self-Ag–MHC class II complexes to developing thymocytes. A recent study showed that medullary TECs presenting promiscuously expressed tissue-restricted Ags under the control of the autoimmune regulator Aire, but not DCs, play an important role in Treg selection (6).

Thymic DCs play a critical role in negative selection of self-reactive thymocytes (7). However, two studies suggest that peripheral DCs can migrate into the thymus and contribute to the induction of nondeletional tolerance (8, 9). We have shown that human thymic DCs conditioned by thymic stromal lymphopoietin expressed by the epithelial cells within Hassall’s corpuscles induce the differentiation of thymocytes into CD4+Foxp3+ Tregs in culture (10). Previous studies have used the bone marrow (BM) chimera class II knockout into wild-type (WT) host to describe the contribution of TECs versus hematopoietic-derived APCs (HCs) in the selection of Tregs and suggested HCs do not play a major role in selection based on the lack of class II on HCs did not decrease the number of Foxp3+ cells generated in the thymus. Another group, using the same model, indicated the contribution of HCs based on a 30% reduction in production of Tregs in the thymus, and, using a thymic transplantation system, they demonstrated that DCs in the periphery can migrate to the thymus, where they induce Treg generation (11, 12).

Our studies differ from previous ones in that we generated BM chimeras in which CD28/B7 signaling was disrupted in either the radiosensitive HCs or radioresistant TECs. Using this model, we were able to discriminate between the function of TECs versus HCs. Our results show that lack of B7 costimulation in both TEC and DC compartments resulted in a significant decrease in the number of Foxp3+ Tregs in the thymus. Restoration of B7 only in HCs induced complete reconstitution of the number of Tregs, indicating that in the absence of functional TECs, HCs have the capacity to induce the development of Tregs. Treg selection by HCs required both class II and B7 expression, indicating that TCR and CD28 signaling cannot be segregated and provided by different APCs in vivo.

Materials and Methods

Mice
C57BL/6, Pep3b (B6.SJL-Pep3b+ Pep3b−/−/−), C57BL/6-J Foxp3GFP, C57BL/6-J H2−−/−J, MHC class II-deficient mice (B6.129-H2−−/−J) on the B6 background, were from The Jackson Laboratory (Bar Harbor, ME). Foxp3GFP reporter mice were provided by F. Xiao-Feng Qin (MD Anderson Cancer Center, Houston, TX). CD11cYFP mice were provided by Michel C. Nussenzweig (The Rockefeller University, New York, NY). All experiments were performed in accordance with federal guidelines approved by the University of Texas, MD Anderson Cancer Center Institutional Care Committee.

The online version of this article contains supplemental material.

Abbreviations used in this paper: BM, bone marrow; DC, dendritic cell; Foxp3, forkhead-winged helix box transcription factor 3; HC, hematopoietic-derived APC; MFI, mean fluorescence intensity; SP, single-positive; TEC, thymic epithelial cell; Treg, regulatory T cell; WT, wild-type; YFP, yellow fluorescent protein.

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BM chimeras
Recipient mice were lethally irradiated (10³ rad). Hematopoietic progenitors were isolated from BM; cells were sorted on the basis of lineage-negative, c-kit+, Sca1+CD45+ after T cell and lineage depletion.Recipient mice were injected i.v. with 1–5 × 10⁶ progenitor cells and maintained on 0.025% Baytril for 2 wk. Chimeric mice were analyzed 8–10 wk postreconstitution.

Abs and flow cytometry
Abs were purchased from BD Biosciences (San Jose, CA), eBioscience (San Diego, CA), and Caltag Laboratories (Burlingame, CA). Intraacellular Foxp3 (eBioscience) and CCL22 (BD Biosciences) staining was done as recommended by the manufacturer. Cells were collected using an LSR II cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR). For thymic DC isolation, thymus was treated with 2 mg/ml collagenase (Life Technologies, Rockville, MD) and 10 μg/ml DNase (Roche, Basel, Switzerland) for 30 min at 37°C. CD4+ cells were depleted by incubating with microbeads, and cells were purified on MACS LD columns (Miltenyi Biotec, Auburn, CA). TECs were isolated by enzymatic digestion as described elsewhere (6). TECs were identified by CD45+ epithelial cell adhesion molecule+ MHC class II+.

Treg suppression assay
Tregs were isolated from spleen and thymus of BM chimeric mice or Foxp3-GFP mice as control by incubation with anti-CD8 microbeads followed by MACS LD columns (Miltenyi Biotec) and sorted on the basis of the CD4+CD25+ or Foxp3+GFP+ Treg phenotype. Cultures were incubated for 72 h, with the inclusion of 1 μg/ml anti-CD3 (2C11; BD Biosciences) in the presence or absence of the indicated ratio of purified CD4+CD25+ or Foxp3+GFP+ Treg. Cultures were incubated for 72 h, with the inclusion of 1 μg/ml anti-CD3 and 1 μg/ml anti-CD25 (2C11; BD Biosciences) in the presence or absence of the indicated ratio of purified CD4+CD25+ or Foxp3+GFP+ Treg. Cultures were incubated for 72 h, with the inclusion of 1 μg/ml anti-CD3 and 1 μg/ml anti-CD25 (2C11; BD Biosciences) in the presence or absence of the indicated ratio of purified CD4+CD25+ or Foxp3+GFP+ Treg. Cultures were incubated for 72 h, with the inclusion of 1 μg/ml anti-CD3 and 1 μg/ml anti-CD25 (2C11; BD Biosciences) in the presence or absence of the indicated ratio of purified CD4+CD25+ or Foxp3+GFP+ Treg. Cultures were incubated for 72 h, with the inclusion of 1 μg/ml anti-CD3 and 1 μg/ml anti-CD25 (2C11; BD Biosciences) in the presence or absence of the indicated ratio of purified CD4+CD25+ or Foxp3+GFP+ Treg. Cultures were incubated for 72 h, with the inclusion of 1 μg/ml anti-CD3 and 1 μg/ml anti-CD25 (2C11; BD Biosciences) in the presence or absence of the indicated ratio of purified CD4+CD25+ or Foxp3+GFP+ Treg. Cultures were incubated for 72 h, with the inclusion of 1 μg/ml anti-CD3 and 1 μg/ml anti-CD25 (2C11; BD Biosciences) in the presence or absence of the indicated ratio of purified CD4+CD25+ or Foxp3+GFP+ Treg.
development of Tregs (Fig. 1E). These data demonstrate that HCs expressing B7 costimulatory molecules are sufficient for the development of Tregs in the thymus in the absence of functional TECs. Also, both TECs and HCs have a comparable capacity to induce the development of Tregs in the thymus.

**MHC class II presentation by HCs is required for generation of Tregs**

Because B7\(^{-/-}\) mice contain a very small population of Tregs in the thymus, the reconstitution of Foxp3\(^{+}\) Tregs in the WT → B7\(^{-/-}\) BM chimera (Fig. 1C) could be the result of Tregs selected by self-Ag presentation by TECs and their subsequent expansion induced by B7 costimulation on HCs. To discriminate between expansion or generation of Tregs mediated by HCs, BM chimeras with class II-deficient, B7-competent HCs and class II-competent but B7-deficient TECs were generated. Irradiated B7\(^{-/-}\) mice were reconstituted with stem cells isolated from BM of MHC class II-deficient mice (class II\(^{-/-}\) → B7\(^{-/-}\)), and the frequency of Tregs generated was compared with WT → B7\(^{-/-}\) and class II\(^{-/-}\) → WT BM chimeras. Eight weeks later, thymocytes from these groups of mice were analyzed by flow cytometry. The results (Fig. 2A, 2C) show that absence of MHC class II in the hematopoietic compartment results in an accumulation of SP CD4\(^{+}\) T cells, confirming the role of HCs in the negative selection of self-reactive T cells (7). Concomitant to the lack of negative selection, the percentages of Foxp3\(^{+}\) Tregs in the class II\(^{-/-}\) → B7\(^{-/-}\) BM chimera were highly reduced (0.2 ± 0.5) as compared with class II\(^{-/-}\) → WT chimeras (2.5 ± 0.8) (Fig. 2A, 2C). Interestingly, lack of negative selection in the class II\(^{-/-}\) → WT BM chimera did not alter the reconstitution of CD4\(^{+}\)Foxp3\(^{+}\) cells in the thymus; those Tregs had similar levels of Foxp3 as WT → WT thymus and were functional in an in vitro suppressive assay (Fig. 2A, Supplemental Fig. 4). These results indicate that competent TECs can compensate as an APC for the development of Tregs, and this process is independent of negative selection mediated by HCs. Similar results were recently reported by Liston et al. (12) postreconstitution of class II\(^{-/-}\) into RAG\(^{-/-}\). In contrast, Proietto et al. (11) demonstrated a 30% reduction in the total numbers of Tregs in the class II\(^{-/-}\) → B7\(^{-/-}\) BM chimera; this difference in Foxp3 T cell numbers may have been attributed to a reduction of thymus size in this BM chimera as mice age (data not shown).

The MHC class II requirement by HCs for the development of Tregs in the B7\(^{-/-}\) mice was also reflected in the total numbers of CD4\(^{+}\)Foxp3\(^{+}\) Tregs within the SP CD4 T cells in the thymus (Fig. 2D). Class II\(^{-/-}\) → B7\(^{-/-}\) BM chimeras had a 70% reduction in Tregs as compared with WT → B7\(^{-/-}\) and class II\(^{-/-}\) → WT BM chimeras, which had competent HCs or TECs as APCs, respectively (Fig. 2D). Interestingly, class II\(^{-/-}\) → B7\(^{-/-}\) BM chimeras had a very small thymus and low numbers of Tregs (Fig. 2D) with no functional suppressive capacity (Supplemental Fig. 4B). Those mice developed severe autoimmune disease characterized by exocrine pancreatitis and gastritis, leading to death of the mice at 12 wk postreconstitution (data not shown). This autoimmune phenotype showed a remarkable similarity to spontaneous autoimmune pancreatitis described in NOD.CD28\(^{-/-}\) mice (13). Overall, these data demonstrate that HCs directly present self-Ags to developing thymocytes and induce the generation of Foxp3\(^{+}\) Tregs in the thymus. In addition, the requirements for TCR signaling and CD28 costimulation in the generation of Foxp3\(^{+}\) Tregs in the thymus cannot be separated in time or space and need to be provided on the same APC type.

**CD4\(^{+}\)Foxp3\(^{+}\) Tregs selected by HCs in the thymus are functional**

To examine the phenotypic and functional characteristics of Foxp3\(^{+}\) Tregs selected by HCs, BM chimeras were constructed by transferring stem cells from WT-Foxp3\(^{GFP}\) mice into lethally irradiated B7\(^{-/-}\) mice (Foxp3\(^{GFP}\) → B7\(^{-/-}\)). Eight weeks later, thymus was collected, and the percentage of CD4\(^{+}\)GFP\(^{+}\) cells was analyzed. As shown in Fig. 3A, the percentage of GFP\(^{+}\)
Data representative of two independent experiments.

**A**

Cytometric analysis of APC stained DCs.

**B**

Flow cytometric characterization of DCs and WT DCs; histogram plots shown from thymocytes from BM chimera (Foxp3GFP → B7−/−) and WT-Foxp3GFP.

**C**

Proliferation of sorted CD4+CD25+ and Foxp3-GFP+ cells and expression of Treg markers as indicated. C. Proliferation of sorted CD4+CD25+ T cells from BL6 spleen (5 × 10^6) incubated with APCs (10^6) and soluble anti-CD3 at the indicated ratio.

**FIGURE 3.** Tregs selected by HCs are functionally suppressive. A. Percentage of SP CD4 T cells and CD4+ Foxp3+ Tregs on BM chimera (Foxp3GFP → B7−/−) and WT-Foxp3GFP. B. Flow cytometric characterization of HC Tregs and WT Tregs; histogram plots shown are from thymocytes from BM chimera (Foxp3GFP → B7−/−) and WT-Foxp3GFP gated on CD4 SP, CD25+, and Foxp3-GFP+ cells and expression of Treg markers as indicated.

**FIGURE 4.** HCs express CD11c and high levels of CD80/86 and chemokine CCL22 costimulation.

**A**

Flow cytometric analysis of enriched CD11c-positive cells isolated from thymus of (CD11cYFP → B7−/−) BM chimera mice (CD11cYFP → B7−/−) and WT-Foxp3GFP mice (2.8 ± 0.4). The two Treg populations (HC selected versus WT) expressed high levels of CD24 and CCR7, which are characteristic of recently differentiated thymic Tregs, and they also expressed high levels of Treg markers such as FR4 and glucocorticoid-induced TNF-like receptor molecules (Fig. 3B).

**B**

Histograms showing the expression of CD8α, CD80/86, and intracellular chemokine CCL22 gated on CD11cYFP, CD11c-APC stained DCs.

**C**

Chemokine assay showing the capacity of isolated HCs and TECs to attract thymocytes in a similar fashion as control chemokine CCL22.

Data representative of two independent experiments.
both hematopoietic and nonhematopoietic APCs for the development of Tregs in the thymus suggests that Tregs selected by these two different APCs may express TCRs that recognize different but complementary repertoires of self-Ags.

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Disclosures

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References


